Current Approaches toward Chemical Mixture Studies at the National Institute of Environmental Health Sciences and the U.S. National Toxicology Program

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The National Institute of Environmental Health Sciences (NIEHS) has several new initiatives involving chemical mixtures and has recognized the need to develop new experimental approaches to enhance our efforts in this area. Responding to recent increases in nominations of complex occupational exposures for toxicologic assessment by the U.S. National Toxicology Program, the NIEHS and the National Institute for Occupational Safety and Health have begun a program to characterize exposures through field studies, identify biomarkers of exposure in workers, and recreate relevant mixed exposures in a laboratory setting. A second initiative with the National Center for Environmental Health/Centers for Disease Control and Prevention will examine blood samples from the U.S. National Health and Nutrition Examination Survey population surveys for selected endocrine-disrupting agents and for common patterns of persistent xenobiotics, providing critical information for the design of animal studies to assess risks of relevant chemical mixtures to humans. New toxicology testing methods (lower cost, faster) will enhance our ability to study chemical mixtures (e.g., dioxin and dioxinlike chemicals, combination AIDS therapies). Ongoing method development efforts involve *in vitro* functional toxicology assays, screens for estrogenic activity, and carcinogenesis studies in transgenic mice. A major scientific initiative with mixtures involves studies of individual and mixtures of dioxin and dioxinlike chemicals to determine if toxic equivalence factors predict carcinogenic potency in traditional and transgenic bioassays. Complementing these studies is an increased emphasis on physiologically based pharmacokinetic modeling, an activity central to the proper interpretation of chemical mixture studies. — *Environ Health Perspect* 106(Suppl 6):1295–1298 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1295-1298bucher/abstract.html*

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There are formidable intellectual challenges associated with the study of chemical mixtures for potential adverse human health effects. These challenges range from the rather mundane problems associated with the creation of stable dose solutions or aerosols of chemical mixtures for delivery to animals or other test systems, to more difficult and serious problems associated with the selection of mixtures for study that provide data relevant to more than one or two unique exposure situations. These and a host of other issues have tended to impede research progress in this area.

During the 1980s rather intense efforts were made at the National Institute of Environmental Health Sciences (NIEHS) to provide data to address some of the fundamental knowledge gaps in studies of chemical mixtures. These efforts were in response to the recommendations of a committee assembled by the National Research Council/National Academy of Sciences following its review of toxicologic and epidemiologic data from studies of well-characterized chemical mixtures (*1,2*). The approach taken was to characterize the toxicity of several well-defined complex chemical mixtures, with plans for follow-up studies to investigate, in depth, factors that accounted for deviations from additivity in toxic responses. Three complex mixtures representing groundwater contamination were chosen for study. One was a 25-chemical mixture design based on the U.S. Environmental Protection Agency (U.S. EPA) groundwater contaminant survey information; two other mixtures mimicked pesticide and fertilizer contamination found in California and Iowa (*3,4*). Although the pesticide/fertilizer studies did not identify toxic effects in experimental animals, there were adverse health findings with the 25-chemical mixture. Subsequent work on this mixture examined the way in which coexposure to low doses of the chemical mixture influenced the acute toxicity of higher doses of known hepatic and renal toxicants (*5*).

More recently, the focus of chemical mixture research at NIEHS has moved away from broad toxicity screening studies of contrived mixtures toward studies of mixed exposures encountered in occupational or environmental settings, and studies of the behavior of simpler mixtures that may share common mechanisms of action. Concurrently, new developments in alternative test models are providing promise that methods will soon be available that will allow us to carry out more studies on selected relevant mixtures in a more cost-effective manner. Some of these new initiatives are described in the following sections.

A new NIEHS/National Institute for Occupational Safety and Health (NIOSH) interagency agreement is supporting research at NIOSH that specifically addresses occupational exposures nominated for toxicological characterization to the U.S. National Toxicology Program (U.S. NTP). Such research will include field studies and/or laboratory re-creation of occupational exposure conditions to determine relevant exposure characteristics and levels, efforts to identify biomarkers of exposure in workers and to evaluate such biomarkers in animal models for use as a cross-species dose metric, and short-term inhalation laboratory studies to identify appropriate exposure generation and measurement approaches and determine relevant responses to exposure.

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Abbreviations used: AZT, zidovudine, NCEH, National Center for Environmental Health; NIEHS, National Institute of Environmental Health Sciences; NIOSH, National Institute for Occupational Safety and Health; PBPK, physiologically based pharmacokinetic; PCB, polychlorinated byphenyl; 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TEF, toxic equivalence factor; U.S. EPA, U.S. Environmental Protection Agency; U.S. NTP, U.S. National Toxicology Program.

This interagency agreement may also support short-term inhalation toxicokinetic studies that may link results from chronic rodent inhalation exposures to single agents with exposures to those agents as complex mixtures. An example of this would be an examination of the relevance of the recent findings of carcinogenicity in rodent studies of an inhaled water soluble cobalt salt (*6*) to occupational exposures to cobalt as it appears in different welding fumes or in the hard metal cobalt alloy industry.

A second interagency agreement that should be extremely helpful in guiding the design of mixture work in the years ahead is under development with the National Center for Environmental Health (NCEH), Centers for Disease Control and Prevention. The NCEH has laboratory and epidemiologic expertise in the measurement of the extent of human exposures to toxic substances and works to determine the levels of exposure that cause human disease. As the principal analytical laboratory working with blood samples from large population-based surveys such as National Health and Nutrition Examination Surveys III and IV, the NCEH has compiled information on the frequency and concentration ranges of many persistent organic chemicals and metals in the U.S. population. Future NIEHS-sponsored activities will include an analysis of the databases to determine whether there are common subsets of chemicals that persist in large segments of the population. These subsets, if they exist, will provide a logical starting point from which further chemical mixture studies can be designed and carried out in rodents. A second major initiative will be to evaluate persistent chemicals found in the population for common biologic activities. For example, many chemicals with rather diverse structures have the capacity to interact with the estrogen receptor. An assessment in an estrogenic activity screen (*7*) of the chemicals routinely found in the human population will allow a determination of whether environmental estrogen exposures are reaching a level of concern in relation to the endogenous levels of these hormones. This information will also allow for studies of relevant combinations of environmental estrogens for possible synergistic activities, as was recently reported by Arnold et al. (*8*).

One of many technical problems that hinders research into chemical mixtures is the difficulty in sorting out variations in responses among animals in basic toxicity screening assays. Although capable of detecting a broad array of toxicologic responses, these assays are perhaps less useful for studying chemical interactions than are those with more biologically discrete end points. An example of a useful new method that is appropriate for chemical interaction studies is the tripartite estrogenicity screen described by Shelby et al. (*7*). This assay comprises competitive *in vitro* binding with the mouse uterine estrogen receptor (presumably both the alpha and newly discovered beta form of the receptor), transcriptional activation in HeLa cells transfected with plasmids containing an estrogen receptor and a response element, and the *in vivo* uterotropic assay in mice. This provides for an assessment of estrogenic activity at three levels of organization and should provide considerable mechanistic insight into an estrogenic or antiestrogenic activity. This assay strategy also allows for a high degree of quantitation of the effects of chemical interactions when studying mixed exposures. The NIEHS gives high priority to studies of persistent organic and inorganic chemicals identified in the human population for their interactive effects in this estrogen activity screen. There is a need for similar approaches for the study of many other hormone-dependent responses. This need was recently made more immediate by the provisions of the Food Quality Protection Act of 1996 (*9*). This act requires the U.S. EPA to analyze, in combination, chemicals such as pesticide residues appearing in food, if those chemicals act through similar mechanisms of action.

Another major technical advance that should provide a stimulus to the evaluation of chemical mixtures for their carcinogenic activity is the recent progress made with transgenic mouse models. These models, particularly two under intense study at the NIEHS, hold promise of providing a means to screen chemicals and chemical mixtures for carcinogenic activity using far fewer animals than the traditional 2-year bioassay, at a much reduced cost, and in studies of 6-months' duration or less.

At a 1995 U.S. NTP Workshop on Mechanism-Based Toxicology in Cancer Risk Assessment: Implications for Research, Regulation, and Legislation (*10*), R. Tennant, NIEHS, presented for discussion the possibility of using genetically altered mice in routine chemical carcinogenicity assessment (*11*). The *p53*-deficient and the Tg.AC transgenic lines were proposed, and currently remain, the primary lines being evaluated as adjuncts to or possible replacements for the rodent bioassay by the NIEHS and the U.S. NTP.

The *p53*-deficient mouse is perhaps one of the more appropriate models for potential carcinogen identification from the viewpoint of a strategy for utilizing transgenic mice. The concept for the use of the hemizygous *p53*-deficient model for detecting potential carcinogens is that the loss of one functional allele would render animals more specifically sensitive to the effects of genotoxic carcinogens. The results of studies to date are encouraging in that it appears that this model is preferentially responsive to mutagenic carcinogens, target organs are similar to those identified in the 2-year bioassays, and the effects are observed after 26 weeks compared to 104 weeks for the standard bioassay.

In contrast to the *p53*-deficient line, the Tg.AC line was created by the germline insertion of a mutated v-Ha-*ras* under the regulation of a fetal zetaglobin promoter sequence and manifests a unique phenotype of wound- and chemical-induced skin papillomagenesis. Studies in the Tg.AC line suggest that the induction of skin papillomas in this mouse model could be used as a reporter phenotype for carcinogens with target organs other than skin in 2-year assays, and based on all studies completed so far, the model is responsive to genotoxic as well as nongenotoxic trans-species carcinogens. There is also some preliminary evidence that other target tissue sites in the Tg.AC line will respond after topical exposure to carcinogens.

The evaluation of transgenic lines by the U.S. NTP/NIEHS is a diverse activity involving a combination of research, standardized testing, and cooperation and interaction with many groups outside the institute. These activities are updated regularly on the U.S. NTP web page (*12*) and are beyond the scope of this report.

As with the estrogen screen, the end points evaluated in the transgenic mouse studies are more discrete or cleaner than those typically evaluated in 2-year bioassays. Although tumors are still the end point of interest, the short duration of the studies and the relatively young age of the animals at the time of sacrifice results in a much lower background of neoplasia or age-related diseases and allows for a more precise determination of treatment-related effects. This is essential to be able to discriminate between chemical interactions that manifest as additive or synergistic using reasonable sample sizes.

In 1993, McLachlan described a research strategy termed functional toxicology (*13*). The purpose was to screen chemicals for common biologic activities using cells transfected with molecular constructs containing specific receptors and reporter genes for that receptor, similar to one part of the estrogen assay described earlier. Batteries of these cells could then be used to screen for a wide variety of receptor-mediated responses to chemicals or chemical mixtures. This approach has the advantage of providing a screening mechanism for chemicals or mixtures with unknown biologic properties, coupled with the ability to provide quantifiable responses. Assays of this type are now commercially available and are being used at the NIEHS under an agreement with the Agency for Toxic Substances and Disease Registry to evaluate a number of pesticides suspected of endocrine-altering activities, and mixtures of common solvents and metals found in groundwater contamination around Superfund waste sites.

When common biologic end points or common modes of action are identified for discrete chemicals, an initial step in studying potential chemical interactions is to standardize the activity of the members of a class of chemicals relative to the most potent member of the class. These standardized responses are termed toxic equivalence factors (TEFs). The approach has perhaps been best developed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) and other dioxinlike chemicals.

The U.S. NTP has a series of 2-year rodent cancer studies underway to determine the extent to which TEFs for dioxin and dioxinlike chemicals predict their cancer-causing potential, target organ specificity, and relative potency. There are several hundred polyhalogenated aromatic compounds that share certain biologic response characteristics with dioxin, or more specifically, 2,3,7,8-TCDD. These include certain polychlorinated biphenyls (PCBs) and polybrominated biphenyls, polychlorodibenzofurans, and other polychlorinated dibenzo-*p*-dioxins. TEFs have been generated for many of these chemicals, primarily through short-term studies of non-cancer end points, comparing their potency to that of dioxin to produce a few or many of the pleiotropic responses to this agent. Although studies to date have indicated a good correspondence of TEFs to cancer promotion in initiation–promotion studies, this has never been adequately tested using the traditional rodent bioassay.

Studies are examining single or combinations of several representatives of the major classes of dioxinlike chemicals found in human adipose tissue. Using the female Sprague–Dawley rat, which is the strain and sex most sensitive to the carcinogenic effects of dioxin and whose response is used as the basis for human risk assessments, studies include 2,3′,4,4′5-pentachlorobiphenyl (PCB 118), 2,3,4,7,8-pentachlorodibenzofuran, and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), as well as 2,3,7,8-TCDD. Also a noncoplanar, nondioxinlike PCB, 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153) is being studied singly and in a binary combination with 2,3,7,8-TCDD to examine possible synergism or antagonism for the carcinogenic effect of 2,3,7,8-TCDD. Finally, a combination of all chemicals other than PCB 153 will be tested as a representative mixture of dioxinlike chemicals. Doses were selected using published information from existing prechronic and chronic rodent studies and the dose ranges were constructed to provide a rigorous test of the relationship between TEFs and carcinogenic potency. Selected tissue concentrations of the chemicals are being determined along with a subset of measures typically used to determine TEFs at various points during the studies such as tissue cytochrome P4501A1 and -1A2 activities, plasma thyroid hormone levels, etc. A related set of studies will evaluate the same chemicals and combinations in the Tg.AC transgenic mouse model. This will provide a rigorous test of the quantitative tumor response for papillomagenesis in comparison with the tumor response in the 2-year rodent assay for this large class of chemicals.

In other NIEHS studies some drugs and drug combinations commonly encountered in acquired immune deficiency syndrome patients are being evaluated for toxicity. Of specific interest are the interactions of zidovudine (AZT) with methadone, rifampin, or sulfamethoxazole/trimethoprim. For this reason, concentration time-course data of AZT, AZT metabolites, and the other drugs are being collected in plasma, liver, vaginal washes, and urine. The Laboratory of Computational Biology and Risk Analysis is applying mathematical methods to the analysis of the pharmacokinetic or pharmacodynamic interactions between AZT and the other drugs. Physiologically based pharmacokinetic (PBPK)/pharmacodynamic models are being developed in association with statistical response surface methodologies for mixture analyses. PBPK models will be tested against the appropriate experimental time course data to identify possible interaction mechanisms.

In summary, U.S. NTP/NIEHS initiatives in the study of chemical mixtures are focusing on complex, real-world exposures that occur in occupational settings or that are identified through blood sampling of the general population. New toxicologic testing methods are providing more cost- and resource-effective ways to study chemical mixtures, and these methods are capable of giving information that is sufficiently quantitative to allow examinations of additive or synergistic actions of mixtures influencing specific biologic activities. Finally, where possible, PBPK models are being used to help unravel the complex kinetic and biologic events that must be sorted out to better understand relationships among exposure to chemical mixtures, target organ dosimetry, and adverse effects.

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